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ON HEREDITY IN CERTAIN MICRO-ORGANISMS, . . *Marshall A. Barber.*

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KANSAS UNIVERSITY SCIENCE BULLETIN.

VOL. IV, No. 1. MARCH, 1907. {WHOLE SERIES, VOL. XIV, No. 1.

ON HEREDITY IN CERTAIN MICRO-ORGANISMS.

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With plates I to IV.

THE aim of the work described in this paper has been to conduct with certain micro-organisms investigations on heredity similar to those long practiced with higher plants and animals. From the offspring of single varying cells new races of yeast and bacteria have been obtained, which differ morphologically and physiologically from the type, and this paper is mainly given to a study of the origin and characteristics of these new races.

In order to accomplish this work, involving as it does the selection and isolation of single varying cells lying among thousands of normal ones, a new method of isolation had to be devised, a method which is described in another part of this paper.

The investigations described below have had to do principally with the yeast *Saccharomyces anomalus* and the bacterium *Bacillus coli communis;* though some work was also done with *B. typhosus* and a large, spore-forming bacillus, probably *B. megatherium.* In every case the work was done with absolutely pure cultures, known to be such because known to be the descendants of single isolated normal cells.

In reviewing the literature on this subject, I shall confine myself largely to those relatively few papers which deal with the heredity of new races which have apparently arisen spontaneously from cells varying independently of the environments, and I shall omit the large number of publications

having to do with modifications of micro-organisms induced by altered conditions of culture, unless such modifications are known or supposed to originate from single varying cells.

Hansen, who has been the pioneer in work of this character conducted on yeasts, succeeded in obtaining asporogenous races of *Sacchoromyces pastorianus* I, II, and III, *S. cerevisiæ* I, *S. ellipsoideus* I and other normally spore-bearing species by plating in gelatin and testing the offspring of various colonies by placing them on gypsum blocks. He found great variation in the spore-producing power of these colonies, varying from normal to races which have remained asporogenous, though cultivated under diverse conditions for twelve years. He found that some varieties, as *johannesberg* II, could not be made to produce asporogenous forms, except by previously cultivating the yeast at temperatures approaching the optimum temperature of budding, and that in practically all forms the per cent. of asporogenous varieties was increased by this preliminary treatment. Here we have to do with a possible transformation associated with selection; but in the case of those forms which gave asporogenous varieties without this treatment, for example *S. pastorianus* I, which gave five to ten per cent. of asporogenous colonies at the start, we deal with probably spontaneous variations.

Associated with the loss of power of producing spores, Hansen found the loss of power to produce veils. *S. anomalus*, however, did not lose this faculty in asporogenous varieties.

Further, the author found types presenting growth of cheesy character and branching filaments, forms which persisted for a number of generations. Again, cells showing a tendency to extend in an elongated mycelium-like form were found to transmit their peculiarities. In the case of *S. ludwigii* the mycelium-like type returned to the normal form when grown in wort. In type No. I of *carlsberg*, Hansen obtained a strain presenting abnormally elongated cells which preserved its peculiarities during two months' cultivation in wort. This type finally returned to its normal form.

A number of races physiologically different which proceed from the same pure culture are mentioned by Hansen. Among these are types showing an increased power of pro-

ducing alcoholic fermentation. A type of *S. cerevisiæ* produced one to three volumes per cent. more alcohol than the parent form from which it was derived.

Other strains differed from their types in their power of forming invertase and maltase. A race of *S. pastorianus* I for some time failed to impart to beer a bitter taste and disagreeable odor characteristic of the type; and variations of other types having to do with the clarification of beer and other aspects of brewing are mentioned by the author.

These physiological types originated for the most part in changed conditions of cultivation. For instance, Biernacki and, later, Märcker, Haydruck and Effront have found that the addition of small quantities of antiseptics to cultures of certain yeasts may increase their fermentative power. But Hansen is of the opinion that in some of these cases, at least, we have to do with a selection of cells endowed with certain physiological characteristics and not with mere transformation. The relative weight of these two factors in such experiments is a matter hard to determine.

M. W. Beijerinck (1897) isolated from a species of *Schizosaccharomyces* found on fruits of tropical origin two sorts of colonies on wort gelatin. One sort, brown in color, was asporogenous; the other, which was white, produced spores, and intermediate forms occurred. On testing the offspring of these colonies, the white variety was found to give nearly all white colonies; the brown gave brown; while the intermediate types gave both white and brown. Certain morphological and physiological differences were also characteristic of the different races. The brown, for instance, divide without forming the characteristic "yokes." The asporogenous race showed a loss of power of producing trypsin. There was little difference in fermentative power, but the chief fermentation was most marked in the spore-bearing form; the secondary, in the asporogenous type.

In *Schizosaccharomyces pombe* this author found white and brown colonies, one of which was more productive of spores than the other. In *S. asporus* he found white colonies with thick, short cells, and brown colonies with similar cells, but having in addition long, thin ones. He found this same

tendency to split into sporogenous and asporogenous races in other species of budding organisms.

Alfred Jörgensen (1898) has described new races, differing in their qualities with respect to brewing, which may come from the offspring of a single cell. This author has successfully selected races having better clearing powers and others with superior aromatic qualities.

H. Will (1899) noted a variation in the types of colonies produced on gelatin by four bottom beer yeasts experimented on. These types varied in the degree of regularity of the colony nucleus and outline. Long growing on one medium tended to fix the type so that fewer variations of this sort were observed. The author found that irregular forms more often occurred on cultures taken from the pellicle, and he noted a parallelism between the tendency to early formation of pellicles and the early formation of outgrowths in colonies. The pellicle of some forms produced irregular colonies, while the sediment of the same type produced regular, though sometimes irregular also. Repeated transfers in wort and beer gelatin tended to restore regularity to the forms of colonies. The outgrowths of irregular colonies were sometimes composed of elongated cells and sometimes of spherical. Spore formation diminished in forms producing irregular colonies and returned again as the colonies became more regular. Races exhibiting cells of mycelium-like form retained their characteristics during three years of repeated transfers in a favorable medium, and the author regards such types as a sort of generation in the cycle of their life-history. He believes that successive phases or generations occur in these plants, and that the reported formation of new races may be only the inception of new generations.

M. Hartman (1903) found in colonies of *Torula colliculosa* on wort gelatin and agar peculiar colonies having elevations composed of cells larger than the normal. Cultures containing these cells have the power of fermenting maltose, a property not possessed by cultures which lack the large cells. Other sugars tested—raffinose, cane, grape and fruit sugars—were fermented by both type and variation alike.

W. Henneberg (1903) found giant cells in each of two types of distillery yeasts, the large size of which was transmitted to daughter-cells budding from them. The tendency to store glycogen was also found to be hereditary in these types.

W. W. Lepeschkin (1903) found in *Schizosaccharomyces pombe* and *S. melacei* cells which grew out in the form of mycelia, instead of dividing in the usual manner of the genus. Some of these cells were isolated and found to reproduce the new characteristic. When grown under conditions favoring endogenous spore formation, these cells produced an oidium-like growth, and spore formation was rarely observed. When spores were produced and made to germinate, they reproduced the elongated type of growth peculiar to the new race. The author thinks the new form an example of mutation or heterogenesis.

Comparatively little has been done in selection experiments on bacteria where the isolation of single cells is involved.

H. W. Conn (1899) describes a culture of bacteria, isolated by him from milk, which shows great variability, not due, apparently, to the immediate environment. The color of colonies varied from a milk white to deep orange, and from colonies rapidly liquefying the medium to non-liquefying colonies. By selection of colonies in plate cultures made from a pure culture he obtained pure white and pure orange, as well as liquefying and non-liquefying strains.

A. Meyer (1901) found that the proportion of branched cells in *Bacillus cohærans* is greater in that part of gelatin plates where branched cells were sown; and he concludes that there is a tendency for this peculiarity to be transmitted. He is of the opinion that bacteria are descended from fungi with branched mycelia, and that occasional branching is to be regarded as atavism, not as the formation of a new character.

W. W. Lepeschkin (1904) found in *Bacillus berestnewii* certain branched individuals and also small non-septate mycelia. The offspring of isolated branched cells exhibited five to fifteen per cent. of branched cells after only twenty to fifty offspring had been formed, while the offspring of the unbranched showed none until after many generations of cells

had formed. A culture coming from an isolated mycelium soon reverted to the ordinary branched and unbranched forms, though the mycelium type persisted long enough to show a tendency to heredity. The appearance of these mycelia, apparently, does not depend on temperature or the nature of the substratum. Higher temperatures seem to favor the appearance of branched forms. The author holds that these variations represent new characteristics and are not to be referred to atavism.

R. Massim (1906), working with a pure culture of *Bacillus coli mutabilis*, found that colonies remained white on Endo agar, indicating lack of power to ferment lactose. Transplantations of young colonies continually gave white colonies on this medium, but transfers from older colonies sometimes gave a proportion of distinctly red colonies, which remained red on further transplantation. These red colonies he supposes to arise by mutation in the sense of de Vries.

I.—EXPERIMENTS ON YEAST.

In my own work on *Saccharomyces anomalus*, I have made use of a culture kindly furnished me by Professor Freeman, of the University of Minnesota, a culture which originally came from Doctor Barker, of England. My researches were conducted in two directions: First, the selection of cells varying from the normal in size; second, the selection of cells varying in form.

In the first series I attempted to obtain a race exhibiting cells permanently larger than the normal by repeated selection of cells of unusual size. As in all experiments made in the course of this work, the series was begun with a pure culture proceeding from a single isolated normal cell. Cultures were made for the most part in glucose bouillon in hanging drops, and the isolated cells were grown in the same medium. In conducting these experiments, a cell, considerably larger than the normal, was isolated, and, after a considerable number, often hundreds, of offspring had been formed, a second large cell was isolated from these, and so on. A check consisting of unselected cells was frequently compared under similar conditions.

In one series this repeated selection was practiced twelve

times; in another, ten times. In both series the results were negative so far as obtaining a permanently modified race is concerned. It was evident in a number of cases that the first few generations proceeding from a large cell consisted of abnormally large cells, but after repeated budding the cell type resumed its normal size. The character of the selected type was noted in hanging drop cultures during the progress of the experiments, and in tube cultures months after the completion of the selection.

In these experiments precautions were taken to avoid reselecting the same large cell in the subsequent selection. A number of single large cells were deprived of their buds and isolated in separate droplets, where they were observed to continue to grow and to reach a size far exceeding the normal and to produce a new crop of buds. On being isolated again, and a second time deprived of their buds, these cells usually refused to form new offspring, and showed an irregularity of outline indicating loss of turgidity and death.

In the above series many cell generations intervened between selections, so that a new series was carried out with another yeast to determine the actual number of generations during which a variation in size persists—a difficult thing to do in an ordinary hanging drop. These experiments were conducted in two ways: First, by means of a very fine glass rod or pipette, but little bent at the tip, a daughter cell was separated from the mother at a time when the attachment showed the relationship clearly. This daughter-cell was isolated, and, when it had grown, its first bud was separated, and so on.

A series several generations long was successfully carried out, but the conditions of the experiment were such that it was difficult to get definite results regarding heredity. Either observations had to be kept up night and day, or growth had to be checked during the intervals in the experiment. This last was accomplished by keeping the hanging drop at refrigerator temperature over night. However, this exposure to lower temperatures, together with the possible injury to cells in the process of separation, subjected them to abnormal conditions; and no very satisfactory results were obtained.

In a second attempt, a single cell was drawn into a capillary tube so fine that budding in two directions only was possible, with the result that a single chain of cells was formed in the tube. It was found, however, that the budding of older cells interpolated new cells in the chain, and it was therefore impossible to keep track of successive generations without keeping the tube under observation night and day. So this attempt was, for the time, also abandoned.

The above experiments indicate that repeated selection is necessary in this yeast if an abnormal standard of size is to be kept up; in this matter the yeast resembles higher plants, where quantitive variations do not often persist unless kept up by continuous selection. It is true that we may conceive of a mutation in the direction of size among lower plants as well as in the higher, but the variations observed in the above experiments did not seem to have that character.

In the second series of experiments, conducted on variations in the form of cells, selections of abnormal cells were continued for some weeks before any variations of a permanent character were obtained. In November, 1903, a cell which showed a narrow, mycelium-like outgrowth, was isolated from a hanging drop of glucose broth culture, the cells of which were the offspring of a single normal cell, isolated the previous day. Growth after isolation was slow, but after one or two days the extension and branching of hypha-like outgrowths produced a mass resembling a small mycelium. This showed little yeast character until after two or three days, when it produced at the tips of branches chains of yeast-cells, which began to reproduce by budding after the manner of yeasts. But the majority of these yeast cells were of a character quite different from the typical form of *Saccharomyces anomalus*. There was a tendency to assume elongated forms, to put out hypha-like prolongations which sometimes branch, and to adhere in groups, characteristics not found in the normal type when grown under like conditions. (See photomicrographs. In plate I, figure 1 represents an old wort culture of the parent stock, figure 2 a new race grown under the same conditions, and figure 3 a ten

days' beef-broth culture of the same new race. In plate II, figures 1 and 2 represent respectively the parent stock and a new race derived from it, both glucose agar cultures, about ten days old, and grown under the same conditions. The new race represented in plate II was originated about November 1, 1903, two years and four months before the time of photographing.)

This new race has persisted three years and five months, and constantly exhibits its new characteristic on a great variety of media, and under very diverse conditions of temperature and amount of oxygen. Some of the media tested were beef-peptone broth, ordinary, and modified by the addition of various sugars, in amounts varying from one-half to ten per cent., plain agar, and agar in combination with glucose or glycerin, wort, wort gelatin, acid and alkaline, glucose gelatin, Loeffler's blood serum, prune juice, and Hansen's fluid medium for yeasts. Both acid and alkaline liquid media were used.

The new characteristics persisted at all temperatures employed, varying from low room temperature to 37½ degrees C., and they were found in cultures of all ages, though they were less marked in cultures a few hours old. The tendency to produce very elongated forms is more marked on gelatin than in liquid cultures, and more pronounced in acid broth than in alkaline.

The tendency of the new race to form elongated cells is well shown on gelatin or agar plate cultures, where the parent type cultivated under these conditions shows for the most part colonies with smooth outlines, while new race colonies show ragged outlines, the irregularities being due to outgrowing, filament-like chains of cells. (See photomicrographs, plate IV. Figure 1, colonies of the parent type grown in glucose gelatin; figure 2, colonies of a new race of the same age and grown under similar conditions.) A new race showed this peculiarity over two years after its origin in as marked a degree as at first.

Colonies of both race and check were obtained in a receptacle from which oxygen had been exhausted by the combustion of phosphorus. These colonies were restored to air,

and after a few days an examination of the new race showed the characteristic elongated form.

The peculiarities of the new race are such that they can be better understood by reference to the illustrations than by statistics. However, in order to get some exact data regarding the elongated character of the cells, I obtained the ratio between the length and breadth of 272 cells of the new race and 212 of the check. In all but 70, measurements were taken of the living cells, in order to avoid error due to shrinking in fixation and staining. Measurements were in nearly all cases made with a $\frac{1}{12}$ oil-immersion objective or with a Zeiss F, and with the micrometer scale in a one-inch ocular. Cells from ten different cultures were taken, with one exception all from glucose broth cultures in hanging drops or from test-tubes. In each of the ten cases, with one exception, the check was of the same age as the new race and grown under the same conditions. Much elongated, filament-like cells were not included in the estimate, and for the most part cells were chosen in which the size or the presence of a well-developed bud showed maturity. Ratios were calculated for each cell separately and the average taken of these ratios. The average ratio of length to breadth was in the check (212 cells) 1.190 to 1.000; in the new race (272 cells), 1.441 to 1.000.

New races of the type described above are characterized by a partial loss of the power to produce spores. Many attempts were made to secure abundant spore formation, among them cultivation on potato, on agar of various sorts, and in shallow hanging drops of various liquid media. Actively growing cultures were also placed on gypsum blocks, moist filter-paper, and on a moist sponge. A considerable range of temperature was employed.

Spores were obtained abundantly in shallow hanging drops in one or two cases, and on glucose agar; but in most of the experiments spores were obtained in relatively small numbers. In all successful spore cultures, with the possible exception of one on glucose agar, there were fewer spores formed in the new races than in the check; and in some instances, where considerable numbers were formed in the check, there

were none at all in the new race cultivated under similar conditions.

Many spores of both race and check were separated from the vegetative cells and isolated in fresh nutrient fluid. In some cases the mother-cell containing ripe spores was isolated, while in others spores were removed from the mother-cells and isolated in separate droplets. The earlier stages of the germination of the spores were usually observed, so as to make sure that the new growth was of spore origin. During a period of nearly two years about fifty such isolations of spores or spore groups were made, of which about one-third were taken from cultures of the new races. Since, in a considerable number of cases, mother-cells with four spores or larger spore groups were included in one isolation, the total number of spores under observation was perhaps 200. A very small proportion of these spores developed further than the formation of a few buds or of a small colony. In some cases a colony of several hundred cells would be formed, but no further development could be obtained. From spores or spore groups isolated from the check not above five permanent cultures were obtained, and from the new races none. It is probable that the culture used had, through long cultivation, partially lost its power of producing healthy spores.

Cultures obtained from spores of the check, whether feeble or permanent, showed a great irregularity in the form and size of cells. These cells closely resemble those from the new races originating in varying vegetative cells, exhibiting much the same elongated form and tendency to group. Besides microscopical differences, these new spore races show macroscopical abnormalities in liquid media. Growth is less vigorous, scanty or no pellicles are formed, and there is a greater tendency for the growth to collect in the bottom of the test-tube, leaving a clear liquid above. A culture originating from spores of the check showed both macroscopical and microscopical abnormalities unimpaired after over two years cultivation on various media.

As stated above, no permanent culture was obtained from spores of the new race, though in one case a colony of about 500 cells was obtained, and in another one of 100 or more.

In both of these the irregular character of the new race was reproduced in the offspring, though the irregularity was scarcely greater than that seen in the offspring of check spores.

A similar tendency towards elongation in cultures from old spores was observed by Hansen in *Saccharoymces ludwigii*.

As regards the formation of pellicles, there seems to be no constant difference between the new races and the check. Pellicles are quickly formed in both, and the cells composing them show the morphological differences characteristic of the two types.

The stability of the new races has been tested not only by three years' cultivation in various media, but also by two series of selection experiments.

In one series an attempt was made to determine whether a new race could be made more filamentous by the selection of the more elongated elements. A single, much elongated cell, usually at least five times as long as broad, or a group of united cells including one or more of such filaments, was isolated, and from its offspring a similar selection made. Eleven such selections were successively made, for the most part in hanging drops of glucose bouillon. It was found that during the experiment there was a greater tendency to a more mycelium-like form of cells, but unless kept up by continuous selection, the filamentous type reverted to the original form of the new race. So there was no evidence that the type could be permanently changed in this direction.

In another series selection was made in an opposite direction. Among the elongated cells of the new races there is almost always found a proportion of spherical cells, not grouping or otherwise visibly differing from the parent type. From a new race, taken about twelve days after its origin, and exhibiting well-marked race peculiarities, selection was made of a single spherical cell, or of a spherical cell with its attached bud, and from the offspring of this cell a similar selection was made. A check of unselected race cells was carried on under parallel conditions. While there was some variation in the degree of sphericity of cells, the check showed the same changes; and at the end of a series of ten

successive selections, the series selected showed the same elongated form as the non-selected check. In other words, the continual selection of cells approaching the parent type brought the new race no nearer to that type.

A long series of experiments was conducted to ascertain the nature of the sports giving rise to permanent new races, their relative proportion to normal cells, and the conditions under which they arise.

The type of cell which most frequently produced permanent new races is characterized by one or more long, narrow prolongations attached to the mother-cell. In hanging drop cultures such cells seemed to appear most frequently in beef peptone broth to which one per cent. of glucose had been added. They appeared in both acid and alkaline broth, though in one long series, at least, they seemed most abundant in the alkaline. They were more often found in shallow hanging drops than in deeper ones, and in obtaining them I had best success by sowing fresh cells in long shallow drops, many of which may be made on one cover-glass. Their more frequent appearance in shallow drops may be in part only apparent, since such drops may be more readily searched. They were found on solid as well as liquid cultures.

In a considerable number of experiments the proportion of these cells relative to normal ones was estimated. Taking two of these experiments for illustration, in acid beef peptone broth containing one per cent. glucose they were found after four days' growth at room temperature in proportions varying from 1 in 5000 in some hanging drops to 1 in 46,000 in others. In roll tubes of glucose gelatin, after six days at room temperature, the proportion varied from 1 in 1000 to 1 in 10,000. On the average, they appeared in numbers less than the proportion 1 in 5000. Sometimes long series of hanging drop cultures, including hundreds of thousands of cells, gave no variations of this character. In the above estimates I refer to comparatively young cultures. On old cultures on solid media the proportion of irregular cells may be somewhat larger.

Most of these variations were found at room temperature. They were found in young as well as old cultures, and in two

instances were found among the offspring of single normal cells isolated on the previous day.

A large number of these cells failed to grow when isolated, and checks consisting of single normal cells isolated and brought to a successful growth under parallel conditions are evidence that the failure to grow is referable to the nature of the abnormal cells and not to the condition of growth. During a period of about two and a half years no less than fifty cells of the typical sport type, or otherwise abnormally elongated, were isolated. From these isolations less than ten new races of permanent character were obtained.

The early development of the new races is slow, and, as stated above, they often exhibit during the first few days a connected mass resembling a mycelium of the higher fungi. But after budding has once freely begun the new races are as vigorous as the type.

About two years after its origin, a new race of *Saccharomyces anomalus* was tested as to its powers of competition with the parent type when mixed with it in cultures. A single cell was isolated from the parent type, and one from the new race, and, after growth had well begun in hanging drops, an approximately equal number of offspring of each cell were mixed and transfers made from the mixture to one per cent. glucose broth in test-tubes, to one per cent. glucose agar, and to hanging drops of glucose broth. After two days' growth transfers were made from each of the three cultures to fresh media of the same kind. These transfers were repeated every two or three days through eight subcultures, the experiment lasting twenty-three days. At the end of this time it was found that the new race had persisted in the hanging drop cultures, had apparently outgrown the parent type in both pellicle and sediment of the broth test-tube cultures, but had so far diminished in the agar cultures that at the margin of the growth it had nearly disappeared and was but little more evident at the center. The hanging drop culture was kept at room temperature; the test-tube cultures at about thirty degrees C.

The agar series was further continued to the fifteenth subculture, and during the last seven transfers it was kept at

room temperature and transfers were made at longer intervals. At the end of this series, extending in all over sixty-five days, cells having the character of the new race reappeared in larger numbers at about the eleventh transfer and continued to increase to the end. As a further test of the persistence of the new race in the agar series, a transfer was made from transfer No. 10 to glucose broth. The new race appeared at once in a considerable proportion of cells, and continued to increase proportionately to the end of a broth series of six transfers. As controls, the parent type and the new race, unmixed, were cultivated on agar and continued through seven transfers at the same intervals and under the same conditions as the series just described. At the end both were found to have retained their characteristics unchanged.

The original mixed broth culture was continued under the same conditions as the agar series to the sixteenth subculture, when the proportion of elongated cells was found to be as large as in an unmixed new race culture kept as a control under parallel conditions. From transfer No. 11 of this mixed broth culture a transfer was made to agar and continued through six agar transfers under the same conditions as the other agar series. The new race characteristics were retained on the solid medium.

In order to confirm the results above given, a second series was begun, starting with mixed new race and parent cells, each proceeding from single cells of the two types, as in the first series. Cultures were continued on both glucose broth and on glucose agar kept at room temperature and transferred six times, at intervals of about five days. In both media the new race persisted as in the first series.

For further confirmation a third series was carried out, this time not beginning with single cells but with a broth culture to which five loopfuls of each type had been added. Transfers were made to glucose broth, plain broth, and glucose gelatin. These were grown at room temperature for twenty-three days and transferred three times. Controls of unmixed cultures of each type were carried through parallel

conditions, a pair for each of the three media. The new race persisted in all media, being least prominent in the gelatin.

Summarizing all experiments with mixed cultures, there is evidence that the new race not only persists in all cultures through as many as sixteen transfers, extending over a period of sixty-five days, but in broth cultures seems to outgrow the parent type. In agar cultures grown at thirty degrees C., with frequent transfers, the new race diminished, but reasserted itself at once on being transferred to broth, and more gradually by continued transfers on agar at room temperature at longer intervals.

Only one strain of *Saccharomyces anomalus*, that consisting of the offspring of a single cell isolated from the culture mentioned at the beginning of this paper, has been employed during the three-year period covered by these experiments; and during this period the type has varied little as regards its capacity of producing sports. There is no evidence of "mutations periods" arising independently of cultural conditions.

Experiments have been begun to ascertain whether variations similar to those found in *Saccharomyces anomalus* occur in other yeasts also. A pink yeast isolated from cider was kept under observation for about a month, and many thousands of cells proceeding from a single isolated cell and grown in shallow hanging drops were searched for variations similar to those which originated new races in *S. anomalus*. Few abnormal cells were found, and these, when isolated, reverted to the parent type. Similar negative results have been obtained from a white yeast isolated from cider and from a large-celled white yeast from dough.

PHYSIOLOGICAL CHARACTERISTICS OF NEW RACES.

New races of the morphological character described above were tested as to their power of fermenting sugars, their power of liquefying wort gelatin, and their resistance to heat and drying.

Since the ordinary fermentation tubes do not give reliable quantitative results, a new form of tube was devised.

This tube (see fig. 1) consists of a glass bulb, *a*, of 25 or 30 cc. capacity, sealed to a U-shaped glass tube, which is

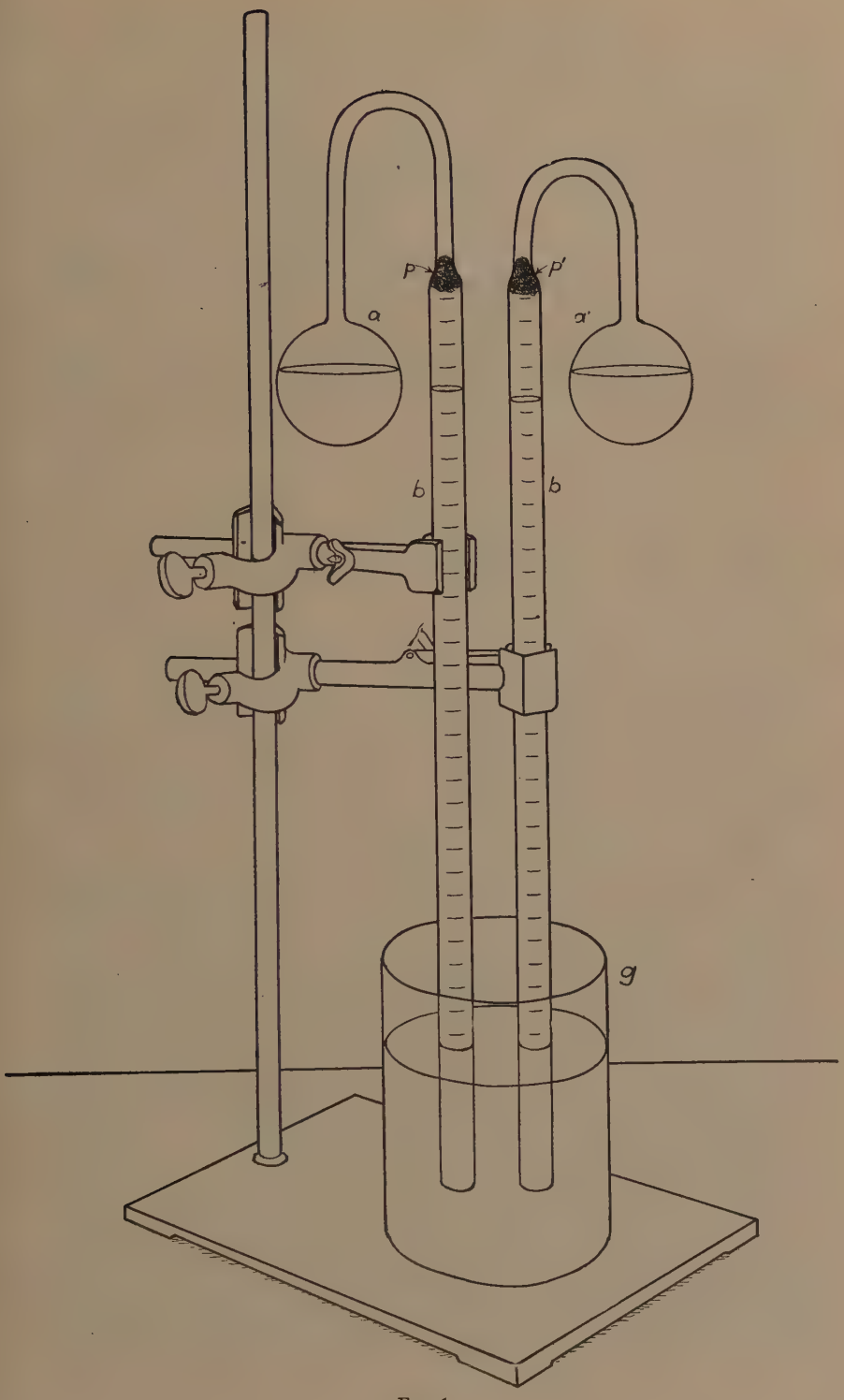

FIG. 1.

sealed at its other end to a burette, *b*, graduated into divisions of $\frac{2}{10}$ of a cubic centimeter. The sugar solution to be tested is introduced by pouring the liquid into the long arm of the apparatus. The end of this arm is then stopped by the thumb, and the apparatus so inclined that the liquid is made to flow into the bulb. The long arm is then plugged with cotton, and the whole sterilized in the autoclave. In inoculating the broth the apparatus is inclined, after removal of the plug of cotton, until a small quantity of the nutrient fluid flows into the connecting neck; and the yeast or bacteria are introduced by means of a platinum loop sealed to the end of a long glass rod or tube. By bringing the apparatus to an upright position again the inoculated portion of broth is made to flow back into the bulb. If this is done carefully no nutrient fluid flows into the graduated arm. A piece of sterilized cotton is then pushed into the graduated arm until it reaches the point, *p*, where the U-connection is attached. The apparatus is then fixed in a clamp and the opening of the long arm placed in water from which air has been recently driven by boiling.

By means of a stiff rubber tube inserted far into the graduated arm, air is exhausted from this arm until water rises in it to a point previously determined. When results from two fermentation tubes are to be compared, this point is at such a level that the same amount of air intervenes in each tube between the top of the water column and the surface of the nutrient liquid in the bulb. Thus the error due to the expansion or contraction of different volumes of air, as the temperature rises or falls, is avoided. In adjusting the water columns to their zero points, the fermentation tubes are raised or lowered in the vessel of water at their base until the two columns of water are of the same height. The fermentation tubes are then placed in an incubator and kept at a nearly constant temperature.

This apparatus was found very satisfactory for comparison of gas production of two types of micro-organisms, since all gas formed is retained, readings are easily obtained, and the apparatus is so compact that the two tubes may easily be placed together in an incubator. Since the whole apparatus

consists of one solid piece of glass, there is no possibility of leakage of air.

In each of the five experiments, the results of which are given in the table below, two similar fermentation tubes were placed together, and one inoculated from a new race culture, the other from a check; and the two cultures supplying material for inoculation were grown under similar conditions long before the inoculation. In every case beef peptone broth, plus the desired per cent. of sugar, was used, and each bulb supplied with broth from the same lot and in the same quantity, always 20 cc. The same new race, one originating about November 1, 1903, was used in all five experiments.

TABLE I.

Date of beginning of experiment, 1905.	Medium.	Max. gas produced by new race, cc.	Max. gas produced by check, cc.	Temperature, degrees centigrade.	Time when maximum was attained.
May 10.....	1% glucose broth.....	14.0	13.6	31–32	2d day.
May 20*....	.....	.....	.....	.....	.....
June 16.....	2% saccharose broth...	36.0	32.1	27–28	2d day.
June 27.....	2% maltose.....	11.2	12.6	27–29	3d day.
July 8.....	2% saccharose	39.1	39.3	23–25	4th day.

*In this experiment broth was used to which lactose had been added after a previous fermentation with *Coli communis*. Good growth was observed in the bulbs, but no gas was formed.

It will be seen from this table that the amount of gas produced by each type was nearly the same except in the two-per-cent. saccharose test of June 16. Several readings were made daily while fermentation was going on actively, and these show that fermentation began in both types at about the same time, rose at nearly the same rate, and reached its maximum at about the same time. After the maximum had been reached, the water in the graduated tube began to rise again, due to the resorption of gas by the water and the nutrient fluid. The rate of resorption was more rapid in the check than in the new race, in all except the one-per-cent. glucose experiment. Since the amount of absorbing surface is practically the same for each tube, and since the check formed as much or more of a pellicle likely to obstruct absorption than the new race, it is probable that in all ex-

cept the glucose experiment fermentation continued longer in the new race.

The amount of gas resorbed by each type approximately seventy-two hours after the maximum was reached is as follows:

TABLE II.

Date of experiment.	Medium.	New race, cc.	Parent type, cc.
May 10	Glucose broth	12.0	4.9
June 16	Saccharose broth	10.6	13.6
June 27	Maltose broth	5.1	8.0
July 8	Saccharose broth	21.7	22.4

The chief aim of these experiments was to compare two organisms; and, while every precaution was taken to keep the two under the same conditions during any one experiment, no especial pains were taken to have the temperature and reaction of medium exactly similar in the different tests. This may explain the discrepancy between the two saccharose tests of June 16 and July 8.

Summarizing, the new race seems to have a greater power of fermentation, but this was not the case in all experiments, nor to any marked extent.

In the experiments bearing on the relative resistance to drying and high temperatures of the two types, the same new race was used as in the fermentation experiments. To determine resistance to drying, cells were subjected to long drying at 35° C. to 40° C., to shorter drying at higher temperatures, or to both. Their resistance to higher moist temperatures was ascertained by exposing them in gelatin or liquefied agar to temperatures ranging from 50° C. to 70° C. for periods of from five to ten minutes. Throughout all these experiments the parent type as a check and new race were exposed to exactly similar conditions. Roll cultures were used for the most part, and a large number of cells sown in each tube.

Of eight dry-heat experiments, two showed small numbers of colonies in the new race, and none in the check. One of these two had been kept at 40° C. to 44° C. for twenty-seven days, the other under the same conditions for one month,

before plating. In a third, exposed to dry heat two days at 37° C., and later brought for a very short time to 50° C., both new race and check produced colonies, but the new race formed them in much greater numbers. Of the five other dry-heat experiments, the new race showed the better growth in four, but the difference was not great. In one, a two-day glycerin agar culture heated six and one-half hours at 43° C. to 45° C., the check showed five colonies, the new race none.

In several moist-heat experiments no growth occurred in either. For instance, no cells survived a temperature of 64° C. to 67½° C. for seven minutes in one experiment, or 70° C. for twenty seconds in another. In three moist-heat experiments growth occurred. In one, a thirteen-day glycerin agar culture heated in gelatin seven minutes at 54½° C. to 57½° C., the new race formed colonies while the check formed none. In a second experiment, in which a four-day culture was heated in glycerin agar to 55° C., there were two series of tubes, one exposed to the high temperature five minutes, the other ten minutes. In both series the new race colonies appeared more abundantly and earlier than in the check. In the ten-minute series only two colonies appeared in the check and these very late. In a third experiment, seven minutes in gelatin at a temperature of 50° C. to 53° C., both types formed colonies, but the new race produced the greater number.

Summarizing the positive experiments of both series, we find six in which the new race surpassed the check to a marked degree, four in which it surpassed but slightly, and one in which the check showed the better growth. These results indicate that the new race has a somewhat greater resistance to heat and drying than the type, in spite of the fact that spore production is greater in the type. As is known, however, yeast-cells not in the spore state may go into a very resistant condition, and it may be such cells which enable the race to withstand the unfavorable conditions. Microscopical examination of the roll cultures showed that only a small per cent. of the cells of either type survived.

As regards the liquefaction of wort gelatin, I have the results of but one experiment. The type and a new race about

two months old were inoculated in both acid and akaline wort gelatin in Miquel flasks, and an additional alkaline wort gelatin series was made in Petri dishes. Both flasks and plates were placed in the dark at room temperature. The results are found in the table given below:

TABLE III.

	After twenty-two days.		After twenty-seven days.	
	Acid wort gelatin.	Alkaline wort gelatin.	Acid wort gelatin.	Alkaline wort gelatin.
Miquel flasks:				
Parent type..	½ to ⅛ liq.....	No liq.....	Wholly liq....	About ⅛ liq.
New race....	Liquefied, but less than ck.	One large liq. colony, but less than on acid gel.....	About $\frac{3}{10}$ liq..	About ½ liq.
Petri dishes:				
Parent type..	.....	Much liq.....	.....	Much liq.
New race ...	.....	No liq.....	.....	Little liq.

From this single experiment little can be deduced except that both types liquefy wort gelatin in nearly the same degree, any difference being in favor of the parent type, and that liquefaction proceeded more rapidly in acid than in alkaline wort gelatin.

The most of the experiments described above were made with a race originated about November 1, 1903. For the sake of confirmation several other new races similar to this one have been isolated from similar varying cells. I have at present in my laboratory four such races of *Saccharomyces anomalus*, ranging from one to three years and five months in age, all of which came from vegetative "sports," and one new race about two years old, arising from a spore. All of these retain their new characteristics apparently undiminished. Checks grown under parallel conditions clearly show that the persistence of the new characteristics is in no wise dependent on the medium or on other conditions of growth.

The results of my work on *Saccharomyces anomalus* may be summed up as follows:

1. Continued selection of cells of more than average size does not permanently modify the type.

2. Variations occur in this species, which, like mutations in higher plants, are capable of giving rise to races endowed

from the beginning with characteristics differing from those of the type. These variations are apparently independent of the immediate conditions of cultivation.

3. New races arising from these variations are characterized morphologically by cells abnormally elongated and tending to adhere in groups, and by a partial loss of the power of producing spores.

4. These morphological characteristics have persisted in cultures continued through three years and five months in a great variety of media, and a new race successfully competes with the parent stock when mixed with it in cultures.

5. Selection in the direction of further modifying the new races or of bringing them back to the type have alike failed to permanently alter the new characteristics.

6. There is evidence that the new races have a greater power of resisting heat and drying, a slightly greater power of fermenting sugars and a somewhat less power of liquefying wort gelatin than the type.

II.—EXPERIMENTS ON BACTERIA.

1. Bacillus coli communis.

In July, 1904, experiments were begun to determine whether the long filaments commonly seen in cultures of *Bacillus coli communis* transmit this character to offspring. A culture kindly supplied by Doctor Fernbach, of the Pasteur Institute, where this part of my work was begun, furnished material for the experiments. In order to secure absolute purity, a single normal cell was isolated at the beginning and experiments were conducted with the progeny of this cell.

A number of long filaments were isolated and failed to grow, but finally one was obtained which began to develop soon after isolation, and gave rise to a race differing morphologically and culturally from the type.

This July, 1904, race will be indicated by the letter A. The principal morphological characteristic of race A is its tendency to form long filaments in a much larger proportion than the type. (See photomicrographs, plate III. Figures 1 and 2 represent the parent type and the new race, respectively, both grown in bouillon under similar conditions.) Under some conditions the culture consists nearly

entirely of these long filaments. The tendency to elongation is more marked in newer cultures than in old, and is at its maximum a few hours after inoculation of tubes. After the culture is a day or two old, whether in hanging drops or test-tubes, its difference from the type becomes less marked, and is evidenced often only by the greater proportion of long filaments, which may be relatively few as compared with those of younger cultures, and, in some cases at least, by the greater length of the shorter filaments as compared with the normal. Young cultures of the race show less motility than the type, due probably to the greater length of filaments. Staining for flagella showed that the new race possesses flagella much like those of the type.

The new race is characterized by macroscopical peculiarities also, especially in plain bouillón cultures at room temperature. These cultures tend to become flocculent, the flocculi often adhering to the sides of the tubes or settling to the bottom, and leaving a comparatively clear liquid in the part of the culture between the pellicle and the sediment. In this respect the new race cultures present a striking contrast with the uniform cloudiness of the check tubes. Cultures of the new race on agar or in glucose bouillon show to the naked eye few or no differences from the type.

On gelatin the characteristics of the new race are strikingly different from those of the parent type. (See plate IV. Figures 3 and 4 represent colonies of parent stock and race A, respectively, grown in gelatin under similar conditions.) The race colonies often show an outgrowth of long filaments. New subcolonies are often formed on these outgrowing filaments forming an irregular group of colonies connected with a larger central one.

Both old and new races readily form pellicles. The pellicles are essentially alike on each, though in some cultures the new race formed pellicles which seemed slightly thicker than those of the type.

The stability of race A has been determined by a long series of subcultures on a great variety of media and under a considerable range of temperature. The filamentous character is more marked in liquid than on solid media, and at

room or refrigerator temperature than in the incubator. But whatever the conditions under which the new race is made to assume a more nearly normal aspect, it returns to the filamentous type when brought into conditions favoring this type; and the new characteristics have persisted unimpaired through a period of two years and eight months' cultivation.

In order to further ascertain the fixity of the new race a selection experiment was carried out. This experiment extended over several days and was conducted in drop cultures. In one series selections were made of the shortest elements of the new race, always selecting cells resembling the normal, and in the other series the longest filaments were selected in a similar manner. When a considerable growth had been obtained the shortest were again selected from the offspring of the short, and longest from the long.

These series were conducted through six selections of the longer filaments and six of the shorter. At the conclusion of the experiments there was no difference apparent between the two types, both having the usual appearance of the new race A. So it is evident that selection from either extreme of the curve of variability does not produce a race of different mean; there was neither accentuation of the peculiarity nor return to the original type.

Indol formation was tested in Dunham's peptone broth, about three months after the origin of race A. The color reaction was approximately the same for each type, and it is concluded that the new race forms indol in about the same degree as the parent form.

One of the most striking cultural characteristics of race A is its increased power of fermenting sugars.

In order to obtain more reliable quantitative results two new sorts of fermentation tubes were devised, one of which (tube No. 1) has already been described under the yeast experiments. In the other sort (tube No. 2) gas is formed under pressure exerted by a column of mercury. The mercury is poured into the apparatus until it enters the lower part of chamber *a* (fig. 2). Nutrient fluid containing the sugar to be fermented is then poured into chamber *a* through

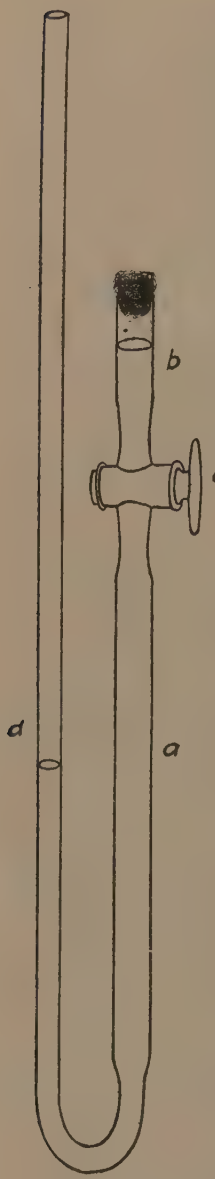

FIG. 2.

the tube *b*. Tube *b* is then plugged with cotton, and the whole apparatus sterilized in the autoclave. The stop-cock at *c* is left open during the sterilization, so that the expanding fluid may rise into tube *b*. After sterilization the stop-cock is closed before the liquid has sufficiently contracted to empty tube *b*. When the apparatus has cooled, the organisms to be tested are introduced into tube *b*, and the stop-cock opened enough to let a portion of the inoculated fluid enter chamber *a*. When gas is formed its pressure is recorded by the mercury column in tube *d*.

This apparatus also makes it possible to compare the growth of organisms in tube *b*, which is freely exposed to the air, with that in *a*, which is under nearly anaerobic conditions.

Three tests of the new race were compared with similar tests of the check in this apparatus, and the results are given in the table below.

In fermentation tube No. 1 gas is formed under negative pressure, and under nearly aerobic conditions. In the *B. coli*, as in the yeast experiments, tubes of this form were always used in pairs, with the new race in one and the check in the other. The same nutrient fluid was used for each, and in the same quantity, 20 cc., and inoculations were made from cultures previously grown under smilar conditions. In the test of July 17, 1905 (see table below), single filaments of the new race and of the type were isolated just before the test, and cultures made from the offspring of these cells were used for inoculation. This was done in order that the inoculated material of each type should

have been as nearly as possible under the same conditions before use.

Examination of the cultures remaining in the bulbs after the close of the experiment was frequently made, and no signs of contamination found.

In order to eliminate possible error due to differences in the fermentation tubes used, the tubes of a pair were sometimes changed after a test, and in a new test each was made to contain the organism previously grown in the other. Both tubes were kept in the incubator at the same temperature during an experiment, and observation was made at the same time of both, and without opening the inner glass door of the incubator.

A possible source of error lies in the fact that there was no maximum recorder in either type of tube. This source of error was largely eliminated by making frequent observations up to the time of maximum pressure.

In the table below, the figures in the second and third columns represent the maximum volume of gas formed in the fermentation tubes of type No. 1, and the the maximum pressure of gas recorded by the mercury column in type No. 2. The hours and average temperatures are reckoned from the time when the apparatus attained the temperature of the incubator until the pressure had reached its maximum.

TABLE IV.—Fermentation tube No. 2. Fermentation under pressure.

Beginning of experiment, 1904.	Mercury, cc.		Time, hours.		Average temperature.	Per cent. excess of A over check.	Medium.
	Type A..	Parent type..	Type A..	Parent type..			
Oct. 31..	18 60	16.50	70	69	28.6	12.7	2% glucose broth.
Nov. 28..	17.15	13.87	125	118	28.0	23.6	Glucose broth about 1½ %.
Dec. 19..	15.85	14.15	150	189	29.0	12.0	Glucose broth about 1½ %.

TABLE V.—Fermentation tube No. 1. Fermentation under partial vacuum.

Beginning of experiment, 1905.	Gas, cc.		Time, hours.		Average temperature.	Per cent. excess of A over check.	Medium.
	Type A..	Parent type..	Type A..	Parent type..			
Jan. 7..	10.80	8.30	43	43	26.6	30.1	2% glucose broth.
Jan. 24..	11.20	8.40	20	20	32.4	33.3	1% glucose broth.
Apr. 19..	15.10	9.90	20	20	36.9	52.5	1% glucose broth.
Apr. 21..	13.60	10.20	18	18	36.0	33.3	1% glucose broth.
Apr. 28..	14.10	10.80	33	33	36 9	30.6	2% lactose broth.
May 5..	14.35	10.60	15	7	37.2	35 4	1% glucose broth.
Jun. 16..	15.20	9.10	24	24	35 9	67.0	2% saccharose broth
May 26..	11.90	5.30	46	23	37.7	124.5	2% saccharose broth
Jun. 27..	13.50	8 20	14	14	36.6	64 6	2% maltose broth.
Jul. 17..	8.50	5.80	17	9	35.5	46.6	2% glucose broth.
Dec. 5..	12.90	11.60	20	24	37.3	11.2	1% glucose broth.

It is seen from the above table that the new race constantly shows a higher maximum gas pressure than the parent type. This is true of all sugars used, though the per cent. of excess is greater in the saccharose and maltose tests than in the glucose. In about half the experiments the maximum pressure was reached at the same time in each type of *B. coli*, while in the others sometimes the one and sometimes the other type reached its maximum first. But there seems to be no correlation between the relative times required to reach the maximum and the per cent. of excess gas pressure in the new type. There is further no constant relation between the temperature and the per cent. of excess gas pressure of the new race.

The gas formed in Smith fermentation tubes by race A and the check was tested and the per cent. of CO_2 found to be approximately the same for each.

Glucose broth cultures inoculated with race A and the check were tested with Fehling's solution after fifty days' growth—about seven days at incubator and about forty-three days at room temperature. Both gave a positive glucose reaction, and in about the same degree.

On Endo agar both race and check produced the characteristic reddening of the medium, and there was no marked difference in the amount of color.

To determine the relative sensibility of the new race and

the parent type to agglutinins, cultures were tested with the serum of two rabbits, the one immunized with gradually increasing doses of new race A, the other immunized in a similar way with the parent type. In both kinds of serum dilutions were made ranging from 1 in 4000 to 1 in 13,000, and in the rabbit immunized with race A, as high as 1 in 21,000. In all these dilutions, the parent race showed decidedly more sensitiveness to agglutinins when fresh broth cultures were used. Agar cultures showed a less decided difference. Tests were made both microscopically and macroscopically; controls were kept, to eliminate error due to spontaneous clumping, and, in order to eliminate the personal equation I submitted the results to two other persons, experienced observers, who independently confirmed my judgment. Many series of tests were made, only one of which I give in the table below. The test was made with the serum of a rabbit inoculated with nine successive inoculations of cultures of race A, the inoculation period extending over twenty-five days and ending with a dose of 3.5 cc. of a six-day broth culture.

TABLE V.

Dilution.	Result after 35–50 minutes.		Result after 2 hours.		Result after 5½ hours.	
	Race A.	Ck.	Race A.	Ck.	Race A.	Ck.
1–14,000.....	?	+	?	+	+	+
1–21,000.....	?	+	?	+	+	+
1–28,000.....	?	?	?	?	?	Slightly?

Thus it is evident that the parent type is more easily agglutinated than the race, whether the agglutinating serum is obtained from an animal immunized to the race or one immunized to the check. This difference between the new type and check may possibly be referred to the more filamentous character of the race, and a consequent less motility. While the difference in agglutination is decided in broth cultures, it is doubtful in emulsions of agar cultures; and it will be remembered that in agar the filamentous character of the race is less pronounced.

The lesions in the inoculated rabbits were insignificant, and there was no decided difference between race and type.

In order to confirm previous results and to ascertain more exactly the sort of variation leading to these new races and the conditions under which they arise, a new series of experiments was undertaken during the summer of 1905. It soon became apparent that variations leading to permanent races are not so common as the early success of the experiments of 1904 had led me to suppose. For nearly six weeks experiments were conducted before a second permanent race was obtained. Under a great variety of conditions, long filaments such as had formed the starting-point of race A were selected, but, on being isolated, they either failed to grow or reverted to the type. Various media, principally plain and glucose broth, were used, in all of which the long filaments were found, and isolations were generally made from young test-tube or hanging drop cultures.

During the period mentioned about 140 filaments of various character were isolated from the original type of *B. coli*, which was the parent of race A (*B. coli*, type I), all except one without success. From another stock of *B. coli* which I isolated from feces (*B. coli*, type II) about fifty more such isolations were made, principally under conditions which had proved successful in obtaining race A, and of these only one new race was obtained. In all, over 190 isolations in long filaments were made, from which only two new races were obtained.

In a number of the above isolations I recorded only failure or success in obtaining a new race, but in 95 I kept a complete record, and the results were as follows: Out of 52 isolations from the original type, *B. coli* I, 36 failed to grow at all, 6 partially developed and failed to grow further, and 9 grew well, but reverted to the parent type, and 1 formed a permanent new race. Of 43 isolations from *B. coli*, type II, 29 failed to grow, 5 showed limited growth, and 8 reverted to the type, and 1 formed a permanent new race. The 95 isolations fairly represent the whole number of attempts made under the best conditions of temperature and medium. The two filaments which grew into permanent new races showed no characteristics differing from the majority found in unsuccessful attempts.

Thus of 95 filaments, approximately 18 per cent. reverted to the parent type, 11 per cent. partially developed, and 68 per cent. failed to grow at all. Two, or about 2 per cent., grew into permanent new races. Including the 11 per cent., the most of which developed only a few threads, with the 68 per cent., we have 79 per cent. which either failed to grow or developed only slightly. Including all filaments isolated in this series, we have of the total number of over 190 filaments only 2, or about one per cent., which formed permanent new races. The number of reversions to type recorded may be too large, because in some experiments selection was made from very young cultures, when there is a greater tendency for normal rods to adhere in filaments than in cultures a few hours older; and it may be that occasionally a filament was selected which was not a variation, but owed its length to its early age merely. In a large proportion of cases, isolations were made of single normal cells under the same conditions to serve as checks. Of these fully eighty per cent. developed. In fact, when these cells were isolated from recent vigorous cultures, as was the case with the larger number of variation isolations, they rarely failed to grow. So the failure of the varitions to grow cannot be laid to the conditions of cultivation or to injury suffered in the process of isolation. It was found that if non-motile filaments were isolated they almost invariably failed to develop further; so, during the latter part of the series of experiments, I isolated for the most part motile filaments alone.

In order to eliminate possible inhibitory effects of concentration of medium in hanging drops an additional series of isolations was made in which long filaments were drawn into capillary tubes immediately after isolation. There were about ten in this series and the results obtained were essentially like those observed in hanging drops.

Among the filaments which reverted to the normal type some produced a progeny which for a time showed an abnormally large number of long filaments, and some of those which only partially developed produced in a few cases four to six long filaments before growth ceased. There seems to

be an almost complete series of gradations between those filaments which showed no growth, and those which immediately reverted to the type. There were, first, filaments which exhibited no growth at all, though in many cases they showed considerable motility after isolation; second, those which merely increased somewhat in length; third, those which produced several filaments or short rods, then ceased to grow further; fourth, those which grew at once into vigorous cultures, but during the first generation showed an abnormally large number of long filaments; and finally those which grew into normal cultures without any peculiarities. The variations which produced permanent new races seem to lie between the third and fourth gradations.

Some of the filaments which partially developed showed some interesting phenomena. The motile filament would divide and produce perhaps one or two long motile filaments, one or two non-motile ones, and perhaps two short, very motile rods. I have found these short rods motile twenty-four hours after the original isolation was made. Their failure to grow cannot be attributed to conditions of culture, since, close beside them, in the same medium and under the same cover-glass, single normal cells of apparently no greater motility rapidly developed numerous offspring.

The new race which I originated during the summer of 1905 from *B. coli*, type I (new race H), exhibits characteristics similar to those of race A, but more closely approaches the type. It has been cultivated nineteen months, on various media, but neither reverts to the type nor approaches race A. I have made three tests of its power of fermentation, using fermentation tubes of the type No. 2. In the first test, made soon after its origin, race H produced a lower maximum than the type. It was then passed through four subcultures, and a second test made five days after the first. It then showed a higher maximum than the type. About four months later it was tested again, and this time showed a maximum 13.7 per cent. greater than the type.

The third new race (race Y), which I obtained in the summer of 1905 from *B. coli*, type II, shows characteristics similar to the other two, but differs less from the type than A and

more than H. While H shows little microscopical difference from its type, Y, like A, tends to form flocculi in bouillon. H has less tendency to form very long filaments than either A or Y, but the average length is much greater than that of the type. I have at present all three of these new races growing in my laboratory, each with a check taken from the type culture at the time when the new race began.

Another sort of new race, characterized by a nearly complete loss of motility, was obtained from an agar culture of the same *B. coli*, type I, from which new races A and H had come. In the water of condensation from this agar culture a series of single cells were isolated, each in a separate droplet. A number of these grew into normal motile cultures; but one of them, which at the time of isolation had the appearance of a normal cell, gave rise to offspring almost wholly non-motile. These did not differ in any other respect from type cells, except that there was a tendency to form groups of short chains lying parallel to one another; and the tendency to adhere in short filaments, seen in all very young cultures, persisted longer in this new race; both tendencies are perhaps due to the loss of motility.

In a large hanging drop from a fresh culture of this non-motile race a few motile cells could usually be seen, and it was found that repeated selections of these motile cells produced cultures of somewhat increased motility. On the other hand, six successive selections of non-motile cells from the original non-motile race has given a type which remains practically non-motile after a period of cultivation extending over nineteen months. Over a year after its origin selections from the very few non-motile cells appearing in cultures of this race have failed to produce a motile type.

Tests have been made of the vigor of growth and the power of fermentation of this non-motile race, and it apparently equals the parent type in these respects. An attempt to restore its motility by repeated transfers of fresh cultures grown under favorable conditions of medium and temperature gave negative results, though seven such transfers were made during a period of three days. Similar transfers of the pa-

rent type under parallel conditions resulted in a comparatively actively motile culture.

Partial loss of motility is a not uncommon phenomenon in the *Colon* group of bacteria, and is often the immediate result of the environment. But the observations described above cannot be all explained as the result of conditions of growth; for the non-motile strains apparently appeared suddenly, and remained non-motile through many generations and under a great variety of conditions; and it seems more probable that we have to do with variations somewhat similar in character to those of race A. But the non-motile types seem less stable and more easily influenced by selection than the type varying morphologically, and it is probable that such non-motile types will in time revert to the parent stock unless kept up by occasional selection.

SUMMARY OF EXPERIMENTS ON COLI COMMUNIS.

1. Variations arise in *Bacillus coli communis*, which, like those of *Saccharomyces anomalus*, may give rise to races exhibiting permanent morphological characteristics not possessed by the type.

2. These variations arise suddenly and apparently independently of conditions of cultivation; and are to be compared with mutants observed in higher plants.

3. They show, in general, a tendency to diminished rapidity of growth at the beginning, but, having once begun to develop, they produce as a rule cultures as vigorous as the normal.

4. They are of different types, and the new races arising from them may be characterized by an abnormal tendency to produce long filaments, or by a nearly complete loss of motility.

5. These new races vary in the degree of their deviation from the type and in their stability. While some apparently require more than one selection to preserve their fixity, others have been constant from the first selection over a period of two years and eight months.

6. One new race further differs from the type in exhibiting an increasing power of fermenting sugars, and a partial loss of sensitiveness to agglutinating serums.

2. Bacillus typhosus.

In the summer of 1904 a series of selections were made of the long threads occurring in cultures of *Bacillus typhosus*, experiments similar to those conducted with *B. coli communis*, and carried on at the same time and under the same conditions. A culture obtained from the collection of the Pasteur Institute was used, and the progeny of a single cell isolated at the beginning furnished material for selection.

A large number of abnormally long filaments were isolated, several of which developed cultures exhibiting more than the normal proportions of long filaments. Of these all but one soon reverted to the type. This one showed the new characteristics for some time and through a number of subcultures; but, being occupied with work on other organisms, I did not follow the history of this race carefully, and it finally died out.

In the summer and autumn of 1905 I resumed these experiments with a new culture of typhoid obtained from Parke, Davis & Co. September 26 I isolated four cells of normal appearance in four separate droplets; three of these developed normal offspring, but one gave rise to a new race characterized by long filaments in much greater proportion than the normal. Colonies on gelatin were much different from those of the parent type cultivated under similar conditions. They were very irregular in form, owing to the outgrowth of long filaments, which formed subcolonies and gave the whole the appearance of a group of small colonies. Bands of parallel thread projected out from a colony, and sometimes curled into peculiar spiral arrangements, owing probably to some resistance met with in their outward progress. Fifty-three days after its origin this culture had apparently reverted to the type.

At the same time that the above typhoid race was isolated, a considerable number of isolations of long threads were made; but all either failed to grow, or, after showing for a time an increased proportion of long filaments, reverted to the type.

These experiments are incomplete, but from them it appears that variations appear in *B. typhosus*, which, when iso-

lated, produce new races similar to those of *B. coli communis*, but of comparatively less stability. Judging from the *B. coli* experiments, however, it is at least possible that by continued selection of long threads one would finally obtain a variation which would produce a permanent new race. In my cultures of *Bacillus typhosus* there was a greater tendency to produce long chains than in *B. coli communis*. As is well known, unselected cultures of *B typhosus* are not uncommonly met with which show a marked tendency to form long filaments, a tendency partially dependent on the medium employed. For instance, in some of my experiments *B. typhosus* showed a marked tendency to form long filaments when grown on agar containing malachite green in the proportion of 1 to 12,000. On being transferred to broth, these cultures reverted to the normal; but the elongated tendency persisted to a slight extent in the first broth culture.

2. Bacillus megatherium (?).

From gum occurring in cane juice obtained from Louisiana I isolated, in December, 1903, a large, plump, motile bacillus, characterized by granular contents, and readily forming spores. From cultures of this bacillus, probably *B. megatherium*, I made many attempts to obtain an asporogenous race by selection. However large the proportion of cells which form spores in hanging drops, there are usually some few which remain motile and sporeless. Scores of those sporeless cells were isolated and cultures obtained from them, usually with negative results as regards obtaining races with diminished spore-forming power. About March 1, 1904, a single sporeless rod of this type was isolated, the offspring of which remained sporeless, though cultivated under conditions under which the type produced spores abundantly. Granules somewhat resembling early stages of spore formation appeared frequently in cells of this race, but no mature spores. At various times during the two months following its origin, this race was compared with the check in broth and on agar. The check formed spores, while the race remained sporeless. Cultures of the sporeless race, taken soon after its origin, and of the check, were sealed in test-tubes. After one year

and nine months the check was found to be still alive, but the new race was no longer viable.

In the summer of 1904 experiments were renewed in the attempt to confirm earlier results, but, though experiments were continued through several weeks, and many series of vegetative rods isolated, all of them reverted to the spore-forming type. These attempts were renewed in the autumn of the same year, and again in the summer of 1905, but with uniformly negative results. The original asporogenous culture of 1904 was continued through many subcultures, but finally died.

While no final conclusions can be founded on the results of one successful experiment, there is good evidence from this experiment that asporogenous races of bacteria, retaining their characteristics for weeks at least, may be obtained by selection of certain vegetative cells. But from the large proportion of failures to obtain new races by the selection of sporeless cells, it is evident that variations which result in asporogenous types are rarely met with.

GENERAL SUMMARY.

In surveying the field in which these experiments lie, one is at once impressed by the similarity between the new races observed here and those arising in higher plants by mutation.

We have in both the sudden appearance of a new type with full-fledged characters arising independently of natural selection, and apparently independently of immediate environment. Successive generations of yeasts or bacteria doubtless find their counterpart in successive cell generations in organs of higher plants; and new races arising among them are to be compared with sports arising vegetatively in multicellular organisms.

While it appears that such variations are much more common among micro-organisms than in higher plants, it may well be that this difference is only apparent, and that there may be very many cell variations in higher plant organs which undergo the same fate as that of hundreds of yeast and bacteria cells which I isolated in my experiments, and either fail to develop or revert to the normal type. There

may be some parallelism between the comparatively few successful new types which I obtained and the sports which become apparent in higher organisms. Whether there is any innate connection generally between a temporarily diminished vegetative activity and heterogenesis, as seemed to be the case with certain of the micro-organisms in my experiments, is, of course, only a matter of conjecture.

We have further this important difference between cell generations in unicellular and higher multicellular plants: the possibility of isolating a single varying cell of the former type alone, a matter which has a bearing on the question whether new races arise among micro-organisms from single varying cells under natural conditions.

The comparative constancy of species of yeasts or bacteria when kept under unvarying conditions argues against the probability that varying cells of the sort which I isolated commonly produce progeny which successfully compete with the parent culture. It will be remembered, too, that a large proportion of these varying cells lack vitality, and in the case of the yeast the growth of the new race is often very slow, until it abandons the more filamentous condition and partially reverts to the type. But there are many chances of accidental isolation of single cells of unicellular plants and in an environment which favors their development; and, once started, they may behave as most of my races did and become as vigorous as the type, and, as in one race tested, they may be capable of competing with the parent race in mixed cultures. Again, there is evidence from the heating and drying experiments conducted on a new race of *Saccharomyces anomalus* that a greater resistance to unfavorable natural conditions may be correlated with a morphological variation. This may be a factor of weight in the origin of new races among micro-organisms, subjected, as many of them are, to great vicissitudes in environment.

So, when we consider that physiological characteristics may be correlated with the morphological, as in the case of the increased power of fermentation in race A of *B. coli communis*, and that we may well have variations characterized by physiological characteristics alone, it seems well within

the range of probabilities that mutations, if such they are, have played some part in the evolution of species of micro-organisms differing physiologically as well as morphologically from the ancestral type. We may have here a factor in the origin or increased virulence of some pathogenic types.

It is suggested by Meyer* that such variations among micro-organisms may be simply a matter of atavism, and Will refers his yeast races to polymorphism. But in experiments on higher organisms as well we often meet with the same difficulty of deciding whether we have to do with the appearance of a new character or the reappearance of a latent one; and in the case of both higher and lower plants only long-continued experiments on many different types can decide the matter. If mutations occur among the cells of higher plants, we would expect, on *a priori* grounds, to find them in the lower also, and perhaps more frequently in these less differentiated and more plastic types.

Fisher (1897) inclines to the view that new races among micro-organisms are to be referred to degeneration. In favor of this view is the diminished vitality of many cells similar to those which originated new races in my experiments, and the slow early growth and diminished spore production of some of the yeast races. But, with the possible exception of *B. coli* Y the new races described above are, when once begun, as vigorous vegetatively as the type, and in the *B. coli* races A, H and Y the offspring of the varying cells early became as vigorous, or nearly as vigorous, as those of the type. Further, these variations appear in relatively few cells and under apparently optimum conditions of growth, so they can hardly be referred to immediate conditions, unfavorable or otherwise, acting on the whole culture.

METHOD OF ISOLATION.†

The essential parts of the apparatus used in the isolations described above consist of an ordinary 1x3 inch glass slip, to which are cemented pieces of glass in such a way as to form a box open at the top and one end (see *b*, fig. 3). The

**Vide supra.*

†A short preliminary description of this method was published in The Journal of the Kansas Medical Society, of November, 1904.

dimensions found most convenient for this box are 40 mm. long by 25 mm. wide by 18 mm. high, though various other dimensions have been successfully used. The sides of the box are lined with filter-paper extending nearly to the top and projecting a few millimeters beyond the open end. A small rod may be inserted at the base of the open end to strengthen the apparatus.

Before making isolations, the filter-paper is thoroughly wet, and a 25x40 mm. cover-glass, previously well cleaned, is sterilized in the flame and placed on the box, to the upper edges of which vaseline has been previously applied. On the sterile under surface of the cover-glass a drop or two of the sterile nutrient fluid to be used is placed by means of a sterilized platinum loop, and near it a drop of the culture containing the organisms to be isolated. The whole is then placed on the stage of the microscope, *a*. A capillary pipette, *b*, is then made by drawing out in the flame a thin-walled glass tube, 8 to 10 cm. in length and about 4 mm. in diameter, in such a way that one end becomes a fine tube, with walls as thin as possible. Holding the thicker end of this tube in one hand and the capillary end with sterilized forceps in the other, the capillary tube is again drawn out over a very small flame, and, just at the moment of drawing, a turn may be given with the forceps so as to form a tip, curved nearly at right angles with the rest of the tube, or the capillary tip may be made straight and afterward bent in the heat of the flame. It is important to have the aperture of this curved tip very small, especially for work with bacteria. I have used pipettes of such dimensions that the opening would admit the smaller yeast cells, but not the larger. If the pipettes are made much smaller than this, capillarity may be so great as to prevent the discharge of liquid from the opening. It is often convenient to make a number of these pipettes, sterilize them in the hot-air sterilizer, and keep them in sterile receptacles ready for use.

In the latest type of this apparatus the thicker part of the pipette is held with a brass holder, clamped on the left side of the stage. The box, held in the mechanical stage with its open end towards the pipette holder, is adjusted so that the

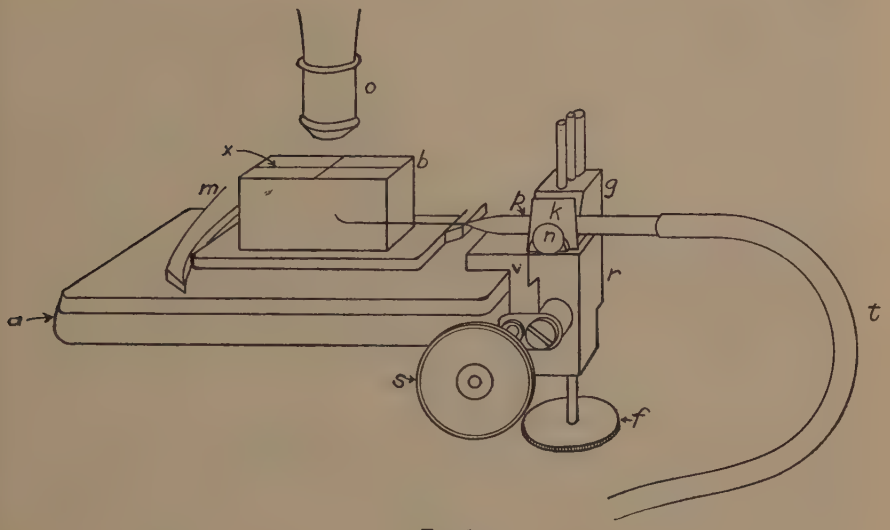

FIG. 3.

cross-lines, x, on the cover are in the center of the field. The pipette is then adjusted by moving it in or out in the groove at the side of g, or laterally by turning the screw, s, which moves parts r and g of the holder. Adjustment is continued until the pipette point is nearly in the center of the field of the microscope. If higher powers are to be used, I usually adjust the pipette under the two-thirds objective first, finally placing it so that its tip is in focus near the center of the field to be used. Finer adjustments may be accomplished laterally by means of screw, s, and in the other direction by a slight movement of the nose-piece holding the objectives. The pipette, together with the parts g, k, and n, holding it, is raised or lowered by means of an adjustment screw, f. The part v is immovable, and remains clamped to the stage of the microscope.

When the pipette is in position it is fixed by the set-screw, n, which tightens clamp k. By means of the mechanical stage that portion of the cover bearing the sterile drop of medium is now brought into the field, the tip of the pipette raised into it and held until it partially fills, or the pipette may be filled before it is placed in the holder by touching its tip to a drop of nutrient fluid held in a sterile platinum loop.

After filling, the pipette is caused to descend and the culture drop brought into the field. The tip of the pipette, which meanwhile is kept in view, is raised until it comes into contact with the micro-organism to be isolated. This immediately enters the pipette by means of capillarity, often in company with other cells; though I have often succeeded in withdrawing the pipette quickly enough to secure only the cell desired. After securing the cells, the cover is moved by means of the mechanical stage until the tip of the pipette can be brought into contact with an unoccupied part of the cover, where its contents are discharged. The discharge is accomplished by blowing gently into a rubber tube, *t*, one end of which is attached to the outer end of the pipette while the other is held in the mouth of the observer. Should there be other cells with the one selected, I usually make a series of droplets with the contents of the pipette, in one of which the cell desired appears alone or with so few other cells that isolation is generally easy at a second attempt. When motile organisms are to be isolated I often place a sterile drop beside the culture drop in such a position that the two drops are in contact at a portion of their circumfer ence. The more active bacteria will soon move into the new drop where they may be taken up by the pipette.

Once isolated, the micro-organism may be picked up and carried to any part of the cover, or, if desired, a fresh sterile cover may be placed on the box and the single cell deposited on it, or, finally, it may be received into a drop on a platinum loop and transferred to a test-tube. In my work I have usually left the isolated cells on the same cover and kept them in view during their development, but in some cases I have drawn the isolated cell well back into the pipette, sealed both ends of the pipette, and placed it in the incubator for development. Many isolations may be made on the surface of one large cover. No difficulty is experienced from the running together of droplets if, just before sterilizing, the cover is rubbed with a piece of chamois skin, or with a cloth having the least trace of vaseline on it. Care must be taken in sterilizing not to leave the cover in the flame long enough to burn off all the vaseline.

After the isolations have been made the cover is sealed over a hollow slide by means of vaseline. I prefer slides with shallow hollows for this, and find that if the hollows are breathed on just before sealing the cover, sufficient moisture will condense on the slide to keep the culture in a saturated atmosphere.

After the cover is safely sealed on the hollow slide the isolations may be examined with higher powers and better light, and any droplets marked so that they may easily be found at later examination. To accomplish this, I generally dip a very fine capillary rod in Brunswick black and with it make one or more dots or lines on the cover above the droplet to be marked. This is easily done under the two-thirds objective after a little practice. It is very helpful to make a large cross with Brunswick black or India ink on the cover before isolating, since the lines serve as a guide for locating droplets. Since the apparatus allows one to control the position of the droplets accurately, they may be arranged thus:

	A	B	C	D
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0

and any droplet recorded by letter and number.

In putting away the culture to develop, one should, of course, put it in a protected place, and avoid placing it so that the cover will be exposed to a lower temperature than the slide beneath, else drops of moisture may condense on the cover and cause the droplets to run together. I use for the most part a special slide with a shallow concavity much larger than that of the ordinary type.

I seldom meet with difficulty from the drying up of droplets during isolation, if the filter-paper, lining the sides of the box, is well wetted before beginning; but, as an additional precaution against both desiccation and contamination, one may nearly close the open end of the box with wetted filter-paper after the pipette has been introduced. If the organism isolated is sensitive to the least concentration of the medium through desiccation, it is well to add fresh sterile nutrient fluid just before sealing.

When the cultures have grown, cells may be removed by means of the same apparatus to a new cover or test-tube; or, if there is room, they may be reselected on the same cover. I have experienced little difficulty from contamination. For many months I have carried on experiments with yeasts and bacteria at the same time, and contamination of the one by the other has been almost unknown, and drops not inoculated uniformly remain sterile. So it seems improbable that yeasts are ever contaminated with other yeasts, or bacteria with bacteria. Even if one could not observe the origin of the yeast and *B. coli* variations, for instance, and follow their first development after isolation, one could hardly attribute the origin of these new races to contamination, considering the small chance that an organism should enter which has so many characteristics in common with the parent culture.

I have described above the later and more elaborate apparatus for isolating micro-organisms. In routine work, unless an objective of higher power than a one-sixth is to be used, I often employ the simpler method first devised. The pipette holder is here dispensed with, and the position of the glass box reversed so that its open end is toward the right. The right hand, steadied by the stage, holds the pipette, and the fine adjustment of the microscope and the mechanical stage are operated by the thumb and second finger of the left. This method has the advantage of simplicity and speed, especially in the matter of changing pipettes; but requires some steadiness of hand and considerable practice in manipulation; for the operation once begun, one cannot lose sight of the tip of the pipette without risking contamination. I find little difficulty, however, in working under the one-fourth and one-sixth objectives with one-inch ocular by this free-hand method.

Besides the work of isolating variations, I have frequently used the above methods as a substitute for plate cultures in isolating organisms. I have successfully isolated single spores of fungi, single cells of algæ, various yeasts, and many bacteria, including *Streptococcus*, from pus. I have found little difficulty in obtaining colonies of amœbæ or infusoria grown from single individuals. The method has some ad-

vantage over the ordinary plate method, in that the process requires much less time, and one can follow the development of a micro-organism from the first, and be sure that the subsequent colony comes from the cell originally isolated. In old cultures a considerable proportion of cells may be dead in the material from which such isolations are made, so it is sometimes necessary to isolate a considerable number of them to obtain a single successful growth. It is possible to work with relatively small quantities of medium in making isolations, and I have obtained sufficient sterile serum for this purpose from small blister made on the hand. It seems probable by this method of isolation something may be done in the way of isolating organisms which are with difficulty handled by ordinary methods. I have as yet made but few experiments in this direction.

The method has been used by several of my students as well as by myself, and they have found it not difficult to acquire. In this description I have omitted many details which would require too much space to describe, but knowledge of these are soon acquired by the experimenter.

I take this opportunity of recognizing the very material assistance rendered me by my students in this work, especially Mr. A. H. Sellards, who has cooperated in many ways, and Mr. Montrose Burrows, who assisted much in designing the pipette holder described above. I wish to acknowledge also the kindness of Doctor Fernbach and other workers at the Pasteur Institute, who placed at my disposal cultures and other laboratory facilities during my work in Paris, in the summer of 1904.

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